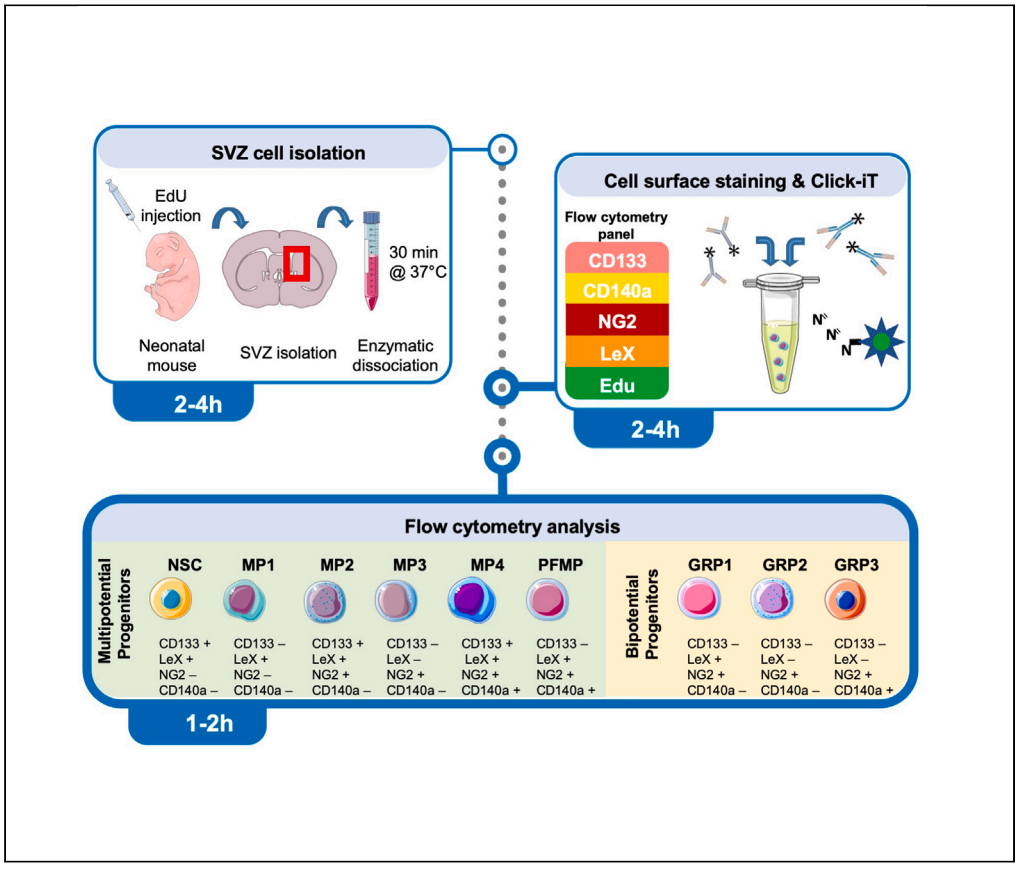
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Analyzing mouse neural stem cell and progenitor cell proliferation using EdU incorporation and multicolor flow cytometry



This protocol describes an *ex vivo* approach to identify and quantify the proportions of proliferating neural stem cells and progenitors of the mouse subventricular zone. It uses ethynyl deoxyuridine (EdU) incorporation to identify dividing cells, combined with multicolor flow cytometry for 4 cell surface antigens to distinguish between 8 phenotypically distinct mouse neural progenitors and stem cells. It has been optimized for wild-type neonatal mice but can be used on mice of any postnatal age.

Fernando Janczur Velloso, Ekta Kumari, Krista D. Buono, Michelle J. Frondelli, Steven W. Levison

fernando.velloso@rutgers.edu (F.J.V.)
levisosw@rutgers.edu (S.W.L.)

Highlights

Protocol to objectively and reproducibly quantify neural stem cells and 7 progenitors

Discerns proliferating SVZ neural progenitors using EdU and 6-color flow cytometry

Optimized for neonatal mice but can be used on mice of any postnatal age

Does not require transgenic mice expressing fluorescent reporters

Velloso et al., STAR Protocols 3, 101065
March 18, 2022
https://doi.org/10.1016/j.xpro.2021.101065

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Analyzing mouse neural stem cell and progenitor cell proliferation using EdU incorporation and multicolor flow cytometry

Fernando Janczur Velloso,[1,3,*] Ekta Kumari,[1] Krista D. Buono,[2] Michelle J. Frondelli,[1] and Steven W. Levison[1,4,*]

[1]Department of Pharmacology, Physiology and Neurosciences, Rutgers-NJMS, Newark, NJ 07103, USA

[2]ICON Laboratory Services, Farmingdale, NY 11735, USA

[3]Technical contact

[4]Lead contact

*Correspondence: fernando.velloso@rutgers.edu (F.J.V.), levisosw@rutgers.edu (S.W.L.)
https://doi.org/10.1016/j.xpro.2021.101065

SUMMARY

This protocol describes an *ex vivo* approach to identify and quantify the proportions of proliferating neural stem cells and progenitors of the mouse subventricular zone. It uses ethynyl deoxyuridine (EdU) incorporation to identify dividing cells, combined with multicolor flow cytometry for 4 cell surface antigens to distinguish between 8 phenotypically distinct mouse neural progenitors and stem cells. It has been optimized for wild-type neonatal mice but can be used on mice of any postnatal age.

For complete details on the use and execution of this profile, please refer to Kumari et al. (2020).

BEFORE YOU BEGIN

There is great interest in studying the stem cells and progenitors of the nervous system, but progress has been hampered because it has been difficult to reliably discern the progenitors from the stem cells. Genetically engineered lines of mice have enabled the stem cells to be analyzed, but this approach limits which mouse strains can be studied or requires extensive breeding to generate a useful line (Beckervordersandforth et al., 2010; Lagace et al., 2007; Yu et al., 2005). By contrast, the technique of flow cytometry, which has been a mainstay of hematopoietic stem and progenitor cell research, exploits the unique antigenic profiles of the stem cells and progenitors and this approach can be used regardless of mouse strain (Schroeder, 2010). Furthermore, flow cytometry allows one to readily study the variety of progenitors that exist simultaneously. In 2007, David Panchision and co-workers combined antibodies against CD133, CD15, CD24, A2B5 and PSA-NCAM, to identify and enrich 4 sets of neural progenitors from the E13.5 and P2 mouse VZ/SVZ. They established that there were multipotent progenitors that could produce neurons, astrocytes and oligodendrocytes, that there were progenitors that were bipotential and either produced neurons and oligodendrocytes, or astrocytes and oligodendrocytes, and that there were progenitors that only produced neurons (Panchision et al., 2007). Extending Panchision's studies we combined CD133 and LeX with CD24, and then added two intermediate progenitor antigens, CD140a and NG2 (Belachew et al., 2003; Chojnacki and Weiss, 2004). As all of the cells examined expressed CD24 and as A2B5 also was widespread, CD24 and A2B5 were eliminated to produce a panel that contained 4 cell surface antigens. With this strategy, 8 phenotypically defined subsets of neural progenitors could be identified within the subventricular zone (SVZ) surrounding the lateral ventricles (Buono et al., 2012). To determine the developmental potential of these 8 subpopulations, SVZ neurospheres were

Table 1. Antigenic profiles identifying SVZ neural stem cell and progenitor populations, frequency in P5 and P20 mouse SVZ and % EdU using two different labeling protocols

Antigenic profile	Population designation	Proportion of total SVZ Cells 5 day old mice[a]	% EdU+[b]	Proportion of total SVZ cells 20 day old mice[c]	% EdU+ (IP)[c]	% EdU+(DW)[c]
CD133+ LeX+ NG2– CD140a–	Neural Stem Cells (NSC)	0.3%	14.6%	0.69%	4.9%	17.8%
NG2- CD140a - LeX+ CD133-	Multipotential Progenitor-1 (MP1)	1.0%	7.0%	0.75%	0.6%	1.3%
NG2+ CD140a - LeX+ CD133+	Multipotential Progenitor- 2 (MP2)	6.1%	17.9%	2.2%	39.9%	47.8%
NG2+ CD140a - LeX+ CD133+	Multipotential Progenitor-4 (MP4)	3.4%	ND	0.22%	42.4%	76.2%
CD133- LeX+NG2+ CD140a +	PDGF-FGF Responsive Multipotential Progenitor (PFMP)	4.0%	8.6%	0.084%	20.3%	47.5%
CD133–LeX+NG2+CD140a–	Bipotential Neuron-Astrocyte Progenitor/Glial Restricted Progenitor-1 (BNAP/GRP1)	9.3%	6.6%	1.5%	8.9%	10.6%
CD133–LeX–NG2+CD140a–	Multipotential Progenitor-3/ Glial Restricted Progenitor-2 (MP3/GRP2)	25%	3.9%	10%	2.5%	0.5%
CD133-LeX-NG2+CD140a+	Glial Restricted Progenitor-3 (GRP3)	4.2%	4.0%	0.37%	1.2%	6.0%
	Other Cells	46.7%		84.2%		

Injections separated by 2 h and initiated 4 h before euthanasia or provided in sweetened drinking water (DW) at 1 mg/mL for 48 h prior to euthanasia.
[a]Note: See Buono et al. (2012).
[b]Note: See Kumari et al. (2020). ND = not determined.
[c]Note: See Frondelli et al. (2021) (EdU was administered as two intraperitoneal (IP) injections).

generated, then separated by FACS, plated onto laminin-coated chamber slides at low density and expanded with growth factors. The multipotential progenitors (progenitors capable of producing neurons, astrocytes and oligodendrocytes) included the NSCs, multipotential progenitors (MP)-1, MP2, MP3, MP4 and the platelet-derived growth factor-fibroblast growth factor responsive (PDGF-FGF)-MP cell (PFMP) (details on their antigenic features are found in Table 1). There were 4 types of bipotential progenitors identified that included the bipotential neuronal-astrocytic progenitor (BNAP) and 3 glial-restricted progenitors (GRP)-1, GRP2 and GRP3. These GRPs produced both "type 1" astrocytes and oligodendrocytes. We have used this method extensively to define how specific growth factors, receptors and injuries affect the composition of the SVZ (Buono et al., 2015a; Chen et al., 2015; Chidambaram et al., 2020; Frondelli and Levison, 2021; Frondelli et al., 2021; Goodus et al., 2015; Kumari et al., 2020; Ziegler et al., 2014, 2019). To further evaluate the proliferation of these neural progenitors the incorporation of the thymidine analogue ethenyl deoxyuridine (EdU) was added to the flow cytometry protocol which is readily compatible with flow cytometry (Buck et al., 2008). Below we describe in detail the protocol that we have used to study the in situ proliferation of the NSCs and progenitors of the SVZ. Note that institutional permission will need to be obtained before this protocol may be used as the protocol requires living mice.

Required solutions and biological samples

Timing: 2 h

1. It is recommended that all solutions be prepared in the afternoon before the experiment (for long-term storage, some solutions may be sterile filtered and kept at 4°C).
2. Prepare stock solutions of DNAse I (1 mg/mL) and Liberase-DH (26 U/mL). These solutions can be aliquoted and frozen at −30°C and stored for at least 2 years.
3. Ensure that a minimum of 5 mice are available from each experimental group, especially if the stem cells are of interest since they represent ~0.2% of the total cell population. 5–15 animals can be pooled for 1 sample. Mice should be ~ 1 week old, preferably between postnatal days 4 and 5.

Note: This protocol has been validated for CD1, Swiss-Webster, and C57Bl/6 mouse strains. To use this protocol with different mouse strains, cell yields may need to be validated.

Table 2. Antibody dilutions and laser configuration for fluorophores

Fluorophore	Dilution	Configuration (Filter:laser)
CD133-APC	1/50	660/20: 633-laser 4
CD140A-PE	1/400	575/26: 488-laser 1
NG2 (unconjugated); Alexa 700 2° Ab	1/50; 1/100	730/45: 633-laser 4
LEX-FITC	1/20	530/30: 488-laser 1
DAPI	1/50,000	450/50: UV-laser 3

This protocol was validated using the fluorophores in this table. Different conjugation options are available for each antibody and can be alternatively used, provided the necessary validation is performed. The configuration in this table was used with a BD LSR II flow cytometer.

⚠ CRITICAL: Mice must be administered ethynyl deoxyuridine (EdU) prior to beginning this procedure. The timing of the EdU administration, number of doses and concentration of EdU may need to be empirically determined. For our studies on the postnatal day 5 mice (P5) we have used a dose of 50 mg/kg based on earlier studies of murine neuroepithelial cells that used bromodeoxyuridine at this dose (Takahashi et al., 1992). A single injection at this dose provided 2 h prior to cell isolation was sufficient to label at least 5% of the cells belonging to each neural progenitor subtype within the SVZ of postnatal day 5 mice (P5) (Kumari et al., 2020). However, for older mice where the cells of the SVZ are less mitototically active we have either administered 2 doses of EdU, separated by 2 h initiated 4 h prior to euthanasia or we have provided the EdU in the drinking water (1 mg/mL in 1% sucrose) where the mice could drink the EdU ad libitum over 48 h. This procedure is both less invasive and produces a significantly greater labeling index as seen in Table 1.

⚠ CRITICAL: This protocol is optimized for cell staining immediately following isolation. However, if a larger cell yield is required, SVZ cells can be propagated *in vitro* as neurospheres prior to staining and analysis (Kumari et al., 2020).

⚠ CRITICAL: All of the antibodies should be titrated before use and the cell concentration and antibody concentrations must be maintained across experiments.

Antibody optimization for flow cytometry

Fluorescence compensation should always be performed. This step can be performed prior to the experiment using compensation beads and the conjugated antibodies (see key resources table).

4. Compensation bead preparation:
   a. Mix the beads tubes and add 1 drop of negative beads and 1 drop of positive beads into a tube with 100 uL of PBS
   b. Add 1 µL of each fluorophore on Table 2.
   c. Mix and incubate protected from light at room temperature (19°C–21°C) for 10 min
   d. Add 1 mL of PGB to the beads and Centrifuge at 1,000×*g* for 5 min.
   e. Remove supernatant and add 150 uL of PBS.
   f. Run the sample in the flow cytometer and perform compensation for each channel (see key resources table).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD133-APC (clone 13A4) (Dilute 1/50)	eBioscience	Cat#17-1331-81; RRID:AB_2734873
CD140a-PE (clone APA5) (Dilute 1/400)	BioLegend	Cat#135905; RRID:AB_1953269

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
NG2 (Polyclonal) (Dilute 1/50)	Millipore Sigma	Cat#AB5320; RRID:AB_91789
LEX (CD15)-FITC(Dilute 1/20)	BD Biosciences	Cat#347423; RRID:AB_10926202
Goat anti-rabbit IgG Alexa fluor 700 (secondary for NG2) (Dilute 1/100)	Thermo Fisher Scientific	Cat#A-21038; RRID:AB_1500674
Rat IgG2a kappa Isotype Control-PE (Dilute 1/400)	eBioscience	Cat#12-4321-82; RRID:AB_470052
Mouse IgM Isotype Control-FITC (Dilute 1/20)	eBioscience	Cat#11-4752-80; RRID:AB_10547648
IgG1 kappa Isotype Control-APC (Dilute 1/50)	eBioscience	Cat#17-4301-81; RID:AB_470177
DAPI (stock of 1 mg/mL) (Dilute 1/50,000)	Sigma-Aldrich	Cat#D9542
Anti-Mouse CD16/CD32 (Fc block) (Dilute 1/50)	Thermo Fisher Scientific	Cat#MFCR00-4; RRID:AB_2539705
Chemicals, peptides, and recombinant proteins		
Liberase-DH	Sigma-Aldrich	Cat#5401054001
DNAse I	Sigma-Aldrich	Cat#4536282001
Fetal Bovine Serum	Sigma-Aldrich	Cat#F2442
32% Paraformaldehyde	Electron Microscopy Sciences	Cat#15714-S
UltraComp eBeads™ Plus Compensation Beads	Thermo Fisher Scientific	Cat#01-3333-41
Dextrose (d-Glucose)	Sigma-Aldrich	Cat#G-7528
Experimental models: Organisms/strains		
C57Bl/6, CD-1 or Swiss Webster mice	Research Animal Vendor	n/a
Critical commercial assays		
Click-iT™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 594	Thermo Fisher Scientific	Cat#C10339
Live/Dead Blue kit	Thermo Fisher Scientific	Cat#L34961
Software and algorithms		
FlowJo	BD Life Sciences	n/a
Other		
Vicell Cell counter	Beckman Coulter	n/a
Dissection tool: 14.5 mm scissors	Fine Science Tools	Cat#14001-14
Dissection tool: #3 scalpel holder	Fine Science Tools	Cat#10003-12
Dissection tool: #10 scalpel blades	Fine Science Tools	Cat#10010-00
Dissection tool: 9 cm scissors	Fine Science Tools	Cat#14060-09
Dissection tool: #5 Dumont fine forceps,	Fine Science Tools	Cat#11295-10
Dissection tool: #7 Dumont curved forceps,	Fine Science Tools	Cat#11297-10
Dissecting scope	Olympus or other	n/a
Shaker (rocker)	Labline	n/a
100μm cell strainer	Falcon	Cat#352360
60 mm dishes	Falcon	Cat#351007
15 mL polypropylene Tubes	Falcon	Cat#352196
LSR II or other similar flow cytometers	BD Biosciences	n/a

Note: The fluorophores conjugated to the antibodies and the fluorophore in the EdU kit in this table can be changed according to the filters and lasers that are available on the flow cytometer. We do not recommend substituting the antibodies that we have specified with other antibodies.

MATERIALS AND EQUIPMENT

Solutions and buffers:

PBS (w/o Ca++ and Mg++)		
Reagent	Final concentration	Amount for 1 L
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na_2HPO_4	10 mM	1.44 g
KH_2PO_4	1.8 mM	0.24 g
ddH_2O	n/a	q.s. 1 L

PBS-Glucose-Mg2+ (PGM)		
Reagent	Final concentration	Amount for 1 L
$MgCl_2$	1 mM	0.2 g
Dextrose	0.6%	6 g
PBS	n/a	q.s. 1 L

PBS-Glucose (PGB)		
Reagent	Final concentration	Amount for 1 L
Dextrose	0.6%	6 g
BSA	0.2%	2 g
PBS	n/a	q.s. 1 L
Storage notes: PBS may be stored at room temperature (19°C–21°C). PBS supplemented with glucose may be autoclaved and then stored at 4°C for up to 1 year.		

Digestion buffer		
Reagent	Final concentration	Amount for 2 mL
Liberase-DH (26 U/mL stock)	0.20 U/mL	15.4μL
DNAse I (1 mg/mL stock in 50% glycerol)	100 μg/mL	20 μL
PGM	n/a	1.96 mL
Use within 1 h after preparing		

Inactivation buffer		
Reagent	Final concentration	Amount for 10 mL
DNAse I (1 mg/mL stock)	100 μg/mL	100 μL
Fetal bovine serum (FBS)	10%	1 mL
PGM	n/a	8.9 mL
Use within 2 h after preparing		

Fixative		
Reagent	Final concentration	Amount for 10 mL
Paraformaldehyde	1%	312.5 μL
PBS	n/a	9.69 mL
Use within 2 h after preparing		

⚠ CRITICAL: Adjust all buffers to pH 7.3 before use.

Storage notes: Liberase and DNAse1 should be aliquoted and stored at −30°C. The aliquots are stable for at least 2 years.

STEP-BY-STEP METHOD DETAILS

SVZ cell isolation

Timing: 2–4 h

Mouse SVZ microdissection followed by chemical and mechanical dissociation.

Note: Use ice-cold buffers and keep cells on ice at all times

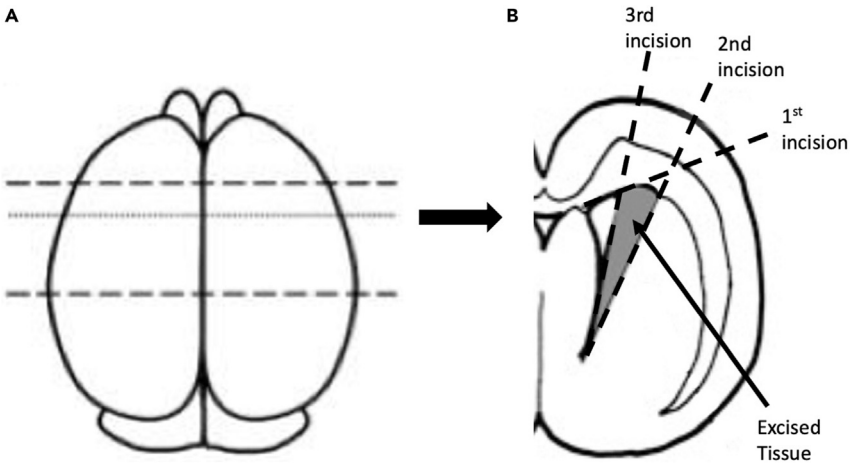


Figure 1. Landmarks for blocking the mouse brain

(A) dotted lines indicate where to cut the brain to create a block for further dissection.

(B) Coronal section of the brain depicts where to make 3 incisions to produce a small piece of periventricular tissue that will contain the dorsolateral and striatal SVZ.

1. Decapitate mouse pup and remove the brain. Transfer the brain to a 60 mm plastic dish or similar surface containing ice-cold PGM.
2. Using a number 10 scalpel, cut coronal sections by making incisions at ~2 mm and ~5 mm from the anterior end of the brain (discard the olfactory bulb) (Figure 1). Move the resulting ~3 mm section to a clean 60 mm plastic dish and keep it submerged in ice-cold PGM.

   Note: Several coronal slices from different animals from the same biological group can be kept in the same plate until the next step.

3. Use curved #7 Dumont forceps to gently move the slices to a dish containing cold PGM. Place the dish under a dissecting scope (with the posterior side facing up).
4. Isolate the area enriched in SVZ cells
   a. Remove the hippocampus to expose the ventricle
   b. Make the 1st incision between the corpus callosum and top of the lateral ventricle
   c. Make the 2nd incision parallel to the wall of the lateral ventricle
   d. Make the 3rd incision parallel to the medial wall of the ventricle
   e. Remove the target region that contains the SVZ (Figure 1) and move it to a clean dish containing ice-cold PGM.
5. Mince the SVZ enriched tissue in PGM into < 1 mm cubes (using #5 straight forceps).
6. Using a wide-bore 1000 μL pipet tip (a wide bore tip can be made by cutting off 2 mm of a standard tip), transfer the tissue to a 15 mL conical polypropylene centrifuge tube, rinsing the dish with PGM to collect all tissue.
7. Centrifuge at 200×*g* for 5 min at room temperature. Remove supernatant.
8. Add 2 mL of **Digestion buffer** and carefully resuspend the pellet.
9. Incubate tube at 37°C under agitation for 30 min (an orbital or rocker shaker at 230 rpm is recommended)

   Note: We have tested other digestive enzyme mixes and found that only Liberase I preserves these antigens on the cell surface. Thus, do not substitute the Liberase I enzyme with any other enzyme. That said, the time of incubation in the Digestion buffer may need to be optimized for the age, strain and tissue volume being used; however, we have found that the digestion protocol defined here works well for both neonatal and adult mouse brains.

10. Add 2 mL of **Inactivation buffer**
11. Centrifuge at 200×*g* for 5 min at room temperature. Remove the supernatant.
12. Shatter the pellet by firmly striking the tip of the tube against the tissue culture hood.
13. Tissue trituration
    a. Add 3 mL of PGB and gently triturate the tissue by pipetting up and down with a P1000 wide bore tip 5 times.
    b. Allow debris to settle down for 5 min
    c. Transfer 2 mL of supernatant to a 50 mL conical tube passing through a 100 μm cell strainer (previously rinsed with PGB).
    d. Add 2 mL of fresh PGB to the original cell tube and triturate using a standard P1000 tip ~15 times (which should be sufficient to dissociate the tissue). Pass all of the solution through a 100 μm filter (cell strainer) into the same 50 mL tube.
    e. Rinse the cell strainer with PGB bringing the volume in the 50 mL conical tube to 25 mL.
    f. Centrifuge at 200×*g* for 10 min at 4°C. Remove supernatant.
    g. Resuspend the cell pellet in 1 mL of PGB. Keep the cells on ice.

Note: If the tissue is not completely dissociated after step 13d, then let the tissue fragments settle, resuspend with 500 mL of PGB and triturate with a P200 tip until dissociated. As debris is created during tissue dissociation, the purpose of increasing the volume to 25 mLs in step 13e above is to reduce the amount of debris collected after centrifugation. Cells should be kept on ice throughout the entire protocol.

Cell surface staining

Timing: 2–4 h

Staining cell suspension with fluorescently conjugated antibodies and using Click-IT chemistry to detect EdU incorporation.

14. Determine the number of viable cells using an automated cell counter or hemocytometer.
15. Transfer 1 × 10^6 viable cells, in 50 μL of PGB, to a 1 mL microcentrifuge tube (if necessary, cells can be centrifuged and resuspended to achieve this cell concentration). This is the sample tube.
16. Prepare 3 additional 1 mL tubes with 1 × 10^5 cells in 50 μL of PGB. These tubes will be used as controls as follow:
    a. Unstained
    b. Isotypes
    c. Live/Dead
17. Add 1 μL of Fc Receptor block (1/50) to each tube and incubate on ice for 10 min
18. Proceed as follows for each tube:
    a. Sample tube:
       i. Add the necessary volumes of each primary antibody to reach the dilutions in Table 2, increasing the final volume to 150 μL of PGB (if more than 1 sample tube is being prepared, prepare a mix of antibodies and distribute an equal volume to each tube).
    b. Isotypes or unstained samples:
       i. Add the same amount of isotype fluorophore controls as used for primary antibodies, increasing the final sample volume to 150 μL of PGB.
       ii. Or omit adding isotypes and increase the volume to 150 uL with PGB
    c. Live/Dead:
       i. Add 100 μL of PGB
       ii. Remove $^1/_2$ of the total volume and incubate at 65°C for 5 min
       iii. Cool on ice
       iv. Return heat-killed cells to original tube (reconstituting the 150 μL in the Live/Dead tube)
       v. Keep on ice until step 25

Note: A commercial live/dead viability stain kit can be used as an alternative to the DAPI staining, which allows the cells to be fixed and then analyzed on the flow cytometer the next day. For compatibility with this protocol, we recommend using a Live/Dead Blue kit for the fluorophores specified in this protocol and to use twice the concentration of the Live/Dead fluorophore as recommended in the manufacturer's instructions. When using Live/Dead fixable kits substitute PGB with PBS in steps 22–27.

19. Incubate all tubes on ice for 20 min (protected from light)
20. Centrifuge at 300×*g* 5 min at 4°C
21. Remove supernatant
22. Resuspend in 200 μL PGB for the first wash.
23. Repeat steps 19–21 for a second wash, resuspending cells in 150 μL PGB
24. Add secondary antibody to sample tube to the dilution in Table 2
25. Add DAPI to all tubes at 1:50,000 dilution
26. Incubate for 25 min on ice (protected from light)
27. Wash 2× with PGB as in steps 19–21
28. Fix cells by adding 1% fresh PFA in PBS w/o Ca2+ or Mg2+ and incubate for 20 min on ice.
29. Centrifuge cells at 300×*g* for 8 min at 4°C and remove supernatant.
30. Resuspend pellet with PBS w/o Ca2+ or Mg2+

Pause Point: At this step, cells can be stored overnight in PBS w/o Ca^{2+} or Mg^{2+}, at 4°C (protected from light).

31. Centrifuge cells at 300×*g* for 5 min at 4°C
32. Discard the supernatant and resuspend cells in 100 μL of 1× Click-It saponin-based permeabilization and wash reagent
33. Incubate for 15 min.
34. Prepare Click-iT reaction cocktail according to the manufacturer's instructions.
35. Add 150 μL of Click-iT reaction cocktail to each well
36. Incubate for 30 min at room temperature, protected from light.
37. Centrifuge cell at 300×*g* for 5 min at 4°C
38. Discard supernatant and wash cells with 200 μL of PGB
39. Centrifuge cells at 300×*g* for 5 min at 4°C
40. Discard supernatant and resuspend pellet in 1 mL of PGB
41. Keep at 4°C until ready to load into flow cytometer

Flow cytometry analysis

Timing: 1–2 h

Running samples through the flow cytometer to evaluate cell phenotype and EdU incorporation.

The flow cytometer must be properly set-up and fluorophore compensation must be performed before analyzing samples. For the fluorophores specified here, the flow cytometer must be configured as described in Table 2 and as described more completely previously (Buono et al., 2015b).

42. Transfer volume of sample, Isotype and Live/Dead tubes to appropriate flow cytometry tubes (depending on the brand and model of the flow cytometry apparatus)
43. Load samples into the flow cytometer applying standard hierarchical gates as illustrated in Figure 2 to:
    a. Remove debris (SSC vs. FSC) (Figure 2A)
    b. Remove doublets (FSC-H vs. FSC-A or SSC-H vs. SSC-A) (Figure 2B)

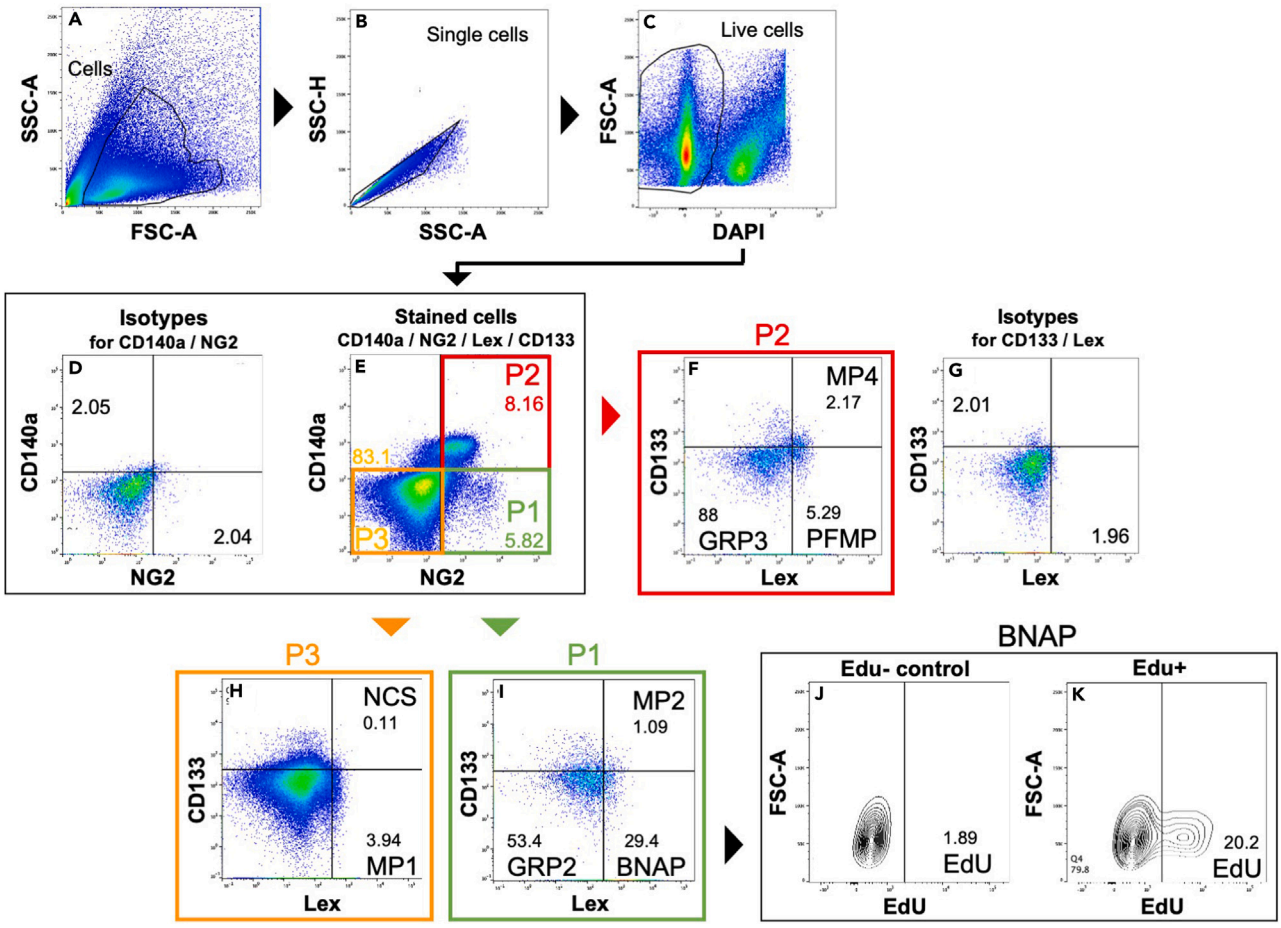


Figure 2. Gating strategy used to study neural progenitors by flow cytometry

SVZs from 12 to 15 mice at P5 were obtained by dissection (Figures 1 and 2), pooled together, dissociated into single cells and then stained for flow cytometry.

(A–C) Single, viable cells were identified using the strategy shown in the panels (A–C). First, cells were separated from debris using side-scatter (SSC-A) versus forward scatter (FSC-A) gating (A). From that gated population single cells were separated from doublets (SSC-H vs SSC-A) (B). Viable cells were then identified based on negative DAPI (or Live/Dead) staining (C).

(E) From these viable cells a double gating strategy was used to classify subpopulations of SVZ neural progenitors. Cells were first characterized based on their expression of CD140a and NG2 (E).

(D) Three subsets P1 (green), P2 (red) and P3 (yellow) were defined based on gates set using isotype controls (D).

(F–I) Next, populations P1 to P3 were individually analyzed for expression of CD133 and LeX (F, H, I), where positively stained cells were, again, gated based on isotype controls (G). This strategy produced the following stem cell and progenitor populations: Within P1: MP2 (CD133+/Lex+), GRP2 (CD133-/Lex-) and BNAP/GRP1 (CD133-/Lex+) (I). Within P2, MP4 (CD133+/Lex+), GRP3 (CD133-/Lex-) and PFMP (CD133-/Lex+) (F). Within P3, NCS (CD133+/Lex+) and MP1 (CD133-/Lex+) (H). The full antigenic profile and frequency of each of these progenitors is provided in Table 1. Proliferative status was evaluated in each population by subsequently gating for EdU incorporation as shown in panel K for BNAP/GRP1s.

(J) Cells unstained for EdU but stained for the other markers (EdU-control) were used to set the gate for EdU+ cells (J).

c. Select live cells by gating on the DAPI negative population. Use Live/Dead (DAPI only) sample to set this gate). (Figure 2C)
d. Use the isotype control tube (or unstained samples) to set the thresholds for cells deemed positively stained for NG2, CD140a, CD15 and CD133 (Figures 2D and 2G).
e. Using the antigenic profiles in Table 1, create gate inclusion criteria for all of the populations listed in Table 1 (Figures 2E, 2F, 2H, 2I and 2J).
f. Using cells from mice that had not received EdU, set the gate for EdU (Figure 2J), then determine the EdU+ cells for each subpopulation of neural progenitors (e.g., for BNAP/GRP1 as shown in Figure 2K).

g. Run all experimental samples, recording at least 100,000 events for each tube. Record data for all required channels.

Note: Isotopes are used to determine the level of background fluorescence due to non-specific binding of the antibodies. Use samples with only the isotype antibodies to define the thresholds for positive cells. We recommend setting the thresholds using the isotype controls to include 2% of the viable cells in the positive gates.

EXPECTED OUTCOMES

For postnatal day 4–5 day old mice, a pool of SVZ enriched tissue from 5 to 7 animals should yield around 2×10^6 viable cells at the start of the staining procedure. All of the cell populations described in Table 1 will be present during the first postnatal week at the frequencies listed. Also, every population is expected to incorporate EdU, but for more slowly cycling cells multiple EdU injections may be necessary as the half-life of EdU in vivo is ~4 h . The relative frequencies of the subpopulation will vary depending on the subject's age. As the neural stem cells (NSC) are a rare population, a higher number of starting cells may be required to detect sufficient numbers of NSCs for analyses.

For further information on the expected proliferative potential and relative abundance of each population refer to Kumari et al. (Stem cell Reports. 2020 May 12;14(5):861-875. https://doi.org/10.1016/j.stemcr.2020.03.019), Buono et al., Dev Neurosci. 2012;34(5):449-62. https://doi.org/10.1159/000345155 and Frondelli et al. (2021) Journal of Neuroscience Research, https://doi.org/10.1016/j.ymeth.2017.08.015.

QUANTIFICATION AND STATISTICAL ANALYSIS

Using a specific software for flow cytometry data analysis (e.g., FlowJo), extract the number of cells in each of the populations in Table 1, for all experimental groups. Note that the gating for specific populations can be performed in this analysis step, provided that the data for all relevant gates was recorded during flow cytometry. Create a one-parameter distinction gate using the fluorophore in the Edu kit to define the proportion of EdU+ (proliferating) vs. EdU- (not proliferating) cells.

These combined data can be used to compare the proportions of the SVZ progenitor and stem cell populations in each sample, as well as evaluate the proportion of proliferating cells in each population. We recommend combining data from at least 3 independent experiments to reach the necessary statistical power using a comparison of means tests.

LIMITATIONS

This protocol has been validated for male mice. Unfortunately, the LeX antigen becomes expressed on telencephalic neurons after E18 in rats, so this protocol may not be useful for studies of rat neural progenitors (Tole et al., 1995), although we have not tested this assumption. We do not expect any significant sex bias in the abundance and proliferative potential of SVZ cell population; however, proper validation should be performed.

TROUBLESHOOTING

Problem 1

Difficulty extracting the SVZ: In Figure 1 we have illustrated the dissection protocol that we use in the lab. However, brain tissue is very soft and it can be difficult making precise cuts so that extra tissue might be included in the tissue extracted for analysis (step 4).

Potential solution

It is not essential to precisely extract the piece of tissue as defined here. The protocol will still work even if the landmarks provided are not exactly followed. However, one should endeavor to extract

only the periventricular region with as little striatal tissue included as possible and to extract the same piece of tissue from each brain to be analyzed.

Problem 2

Low cell yield: We have recommended that each experimental sample be adjusted to 1×10^6 cells before the surface staining step. Lower numbers of cells at this step may reflect a problem during the cell dissociation (step 15).

Potential solution

Most commonly, low cell yield arises from cell death during the enzymatic dissociation process. The time of incubation in the dissociation buffer can be adjusted for the specific age, strain and number of animals in each experiment. Incubation time in the dissociation buffer should be as short as possible to avoid cell death. Also, the mechanical dissociation step (trituration) should be performed as gently as possible. If necessary, collect samples from the cell suspension immediately after the enzymatic dissociation. It is important at this step to add DNase to prevent and limit aggregation. After the mechanical dissociation, one can evaluate the total number of viable cells to determine the number of aggregates and single cells in each sample to assess which step needs to be optimized. Optimizing centrifugation time and speed is also important to maximize cell yield. Centrifugation speed should not be too slow and/or too short as the cells won't pellet, nor should it be too fast and/or too long to prevent the cells from forming a tight pellet. In fact, the latter might result in a greater cell loss because the pellet will be difficult to dissociate. A modification to reduce cell loss is to use 96 well V-bottom plates to stain the cells to avoid disperse, loose cell pellets. For analyzing cells in the adult mouse brain, double the Liberase concentration and centrifuge through 22% Percoll to reduce debris. See Buono et al. (2012).

Problem 3

Low viability (step 13).

Potential solution

Optimize the time with the digestion enzymes, use optimized centrifugation forces and time and don't leave the samples unattended after centrifugation. Gentle and thorough wash steps will help to remove debris coming from the tissue.

Problem 4

Too much debris. If there is too much debris in your sample it will bind the antibodies reducing the strength of the fluorescent signal and increasing the levels of autofluorescence. Also, if there is too much debris it will create artifacts that will make it difficult to set the gates properly (step 13).

Potential solution

To reduce the amount of debris in your samples you may need to adjust the enzyme incubation time and the ratio of the enzyme to tissue. The 25 mL volume can be brought to 50 mLs to further reduce debris collection. Also, as stated above, you may add a centrifugation step through 22% Percoll to reduce debris. When analyzing the data, make sure that the FSC is increased to have a good separation of debris from the cells.

Problem 5

Edu Click-iT kit reagent interfering with fluorophores in the flow cytometry panel (During Analysis).

Potential solution

The Click-iT reaction can interfere with the fluorescence of certain fluorophores. If the fluorophores specified in this protocol are changed to different fluorophores then you may not see some of the expected cell populations. Therefore, perform a pilot staining experiment with appropriate controls

to see if all the cell populations are captured after performing this flow cytometry using different fluorophores.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Steven W. Levison (levisosw@rutgers.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This protocol did not generate datasets or code.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health #s R01NS116828-01 and R21NS107772 awarded to S.W.L. and by CAUT22AFP009 awarded to F.J.V. awarded by the NJ Governor's Council on Autism. We are grateful to Dana Stein, Dr. Suki Singh, Dr. Doug Wilson, and Tammy Galenkamp in the New Jersey Medical School Flow Cytometry core facility for all of their assistance, advice and guidance and to past members of the Levison Lab who assisted us in optimizing this protocol.

AUTHOR CONTRIBUTIONS

K.B. optimized the flow cytometry protocol for the 4 antibodies against the cell surface antigens. E.K., M.J.F., and F.J.V. optimized the method for EdU detection. All authors participated in writing this protocol and have reviewed the manuscript. S.W.L. participated in the design of the protocol, supervised the execution of the experiments, oversaw the data analysis and performed final edits of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests. E.K. is presently a Senior Research Investigator at the Incyte Research Institute in Wilmington, DE.

REFERENCES

Beckervordersandforth, R., Tripathi, P., Ninkovic, J., Bayam, E., Lepier, A., Stempfhuber, B., Kirchhoff, F., Hirrlinger, J., Haslinger, A., Lie, D.C., et al. (2010). In vivo fate mapping and expression analysis reveals molecular hallmarks of prospectively isolated adult neural stem cells. Cell Stem Cell *7*, 744–758.

Belachew, S., Chittajallu, R., Aguirre, A.A., Yuan, X., Kirby, M., Anderson, S., and Gallo, V. (2003). Postnatal NG2 proteoglycan-expressing progenitor cells are intrinsically multipotent and generate functional neurons. J. Cell Biol *161*, 169–186.

Buck, S.B., Bradford, J., Gee, K.R., Agnew, B.J., Clarke, S.T., and Salic, A. (2008). Detection of S-phase cell cycle progression using 5-ethynyl-2′-deoxyuridine incorporation with click chemistry, an alternative to using 5-bromo-2′-deoxyuridine antibodies. Biotechniques *44*, 927–929.

Buono, K.D., Goodus, M.T., Guardia Clausi, M., Jiang, Y., Loporchio, D., and Levison, S.W. (2015a). Mechanisms of mouse neural precursor expansion after neonatal hypoxia-ischemia. J. Neurosci. *35*, 8855–8865.

Buono, K.D., Goodus, M.T., Moore, L., Ziegler, A.N., and levison, S. (2015b). Multimarker flow cytometric characterization, isolation and differentiation of neural stem cells and progenitors of the normal and injured mouse subventricular zone. In Neural Surface Antigens: From Basic Biology towards Biomedical Applications, J. Prusac, ed. (Elsevier Press), pp. 175–185.

Buono, K.D., Vadlamuri, D., Gan, Q., and Levison, S.W. (2012). Leukemia inhibitory factor is essential for subventricular zone neural stem cell and progenitor homeostasis as revealed by a novel flow cytometric analysis. Dev. Neurosci. *34*, 449–462.

Chen, H., Goodus, M.T., de Toledo, S.M., Azzam, E.I., Levison, S.W., and Souayah, N. (2015). Ionizing radiation perturbs cell cycle progression of neural precursors in the subventricular zone without affecting their long-term self-renewal. ASN Neuro *7*, 1–16.

Chidambaram, S., Velloso, F.J., Rothbard, D.E., Deshpande, K., Cajuste, Y., Snyder, K.M., Fajardo, E., Fiser, A., Tapinos, N., Levison, S.W., et al. (2020). Subventricular zone adult mouse neural stem cells and human glioblastoma stem cells require insulin receptor for self-renewal. bioRxiv. https://doi.org/10.1101/2020.03.10.985598.

Chojnacki, A., and Weiss, S. (2004). Isolation of a novel platelet-derived growth factor-responsive precursor from the embryonic ventral forebrain. J. Neurosci. *24*, 10888–10899.

Frondelli, M.J., and Levison, S.W. (2021). Leukemia inhibitory factor is required for subventricular zone astrocyte progenitor proliferation and for prokineticin-2 production after a closed head injury in mice. Neurotrauma Rep. *2*, 285–302.

Frondelli, M.J., Mather, M., and Levison, S. (2021). Oligodendrocyte progenitor proliferation is disinhibited following traumatic brain injury in leukemia inhibitory factor heterozygous mice. J. Neurosci. Res., In Press. https://doi.org/10.1002/jnr.24984.

Goodus, M.T., Guzman, A.M., Calderon, F., Jiang, Y., and Levison, S.W. (2015). Neural stem cells in the immature, but not the mature, subventricular zone

respond robustly to traumatic brain injury. Dev. Neurosci. *37*, 29–42.

Kumari, E., Velloso, F.J., Nasuhidehnavi, A., Somasundaram, A., Savanur, V.H., Buono, K.D., and Levison, S.W. (2020). Developmental IL-6 exposure favors production of pdgf-responsive multipotential progenitors at the expense of neural stem cells and other progenitors. Stem Cell Rep. *14*, 861–875.

Lagace, D.C., Whitman, M.C., Noonan, M.A., Ables, J.L., DeCarolis, N.A., Arguello, A.A., Donovan, M.H., Fischer, S.J., Farnbauch, L.A., Beech, R.D., et al. (2007). Dynamic contribution of nestin-expressing stem cells to adult neurogenesis. J. Neurosci. *27*, 12623–12629.

Panchision, D.M., Chen, H.L., Pistollato, F., Papini, D., Ni, H.T., and Hawley, T.S. (2007). Optimized flow cytometric analysis of central nervous system tissue reveals novel functional relationships among cells expressing CD133, CD15, and CD24. Stem Cells *25*, 1560–1570.

Schroeder, T. (2010). Hematopoietic stem cell heterogeneity: subtypes, not unpredictable behavior. Cell Stem Cell *6*, 203–207.

Takahashi, T., Nowakowski, R.S., and Caviness, V.S., Jr. (1992). BUdR as an S-phase marker for quantitative studies of cytokinetic behaviour in the murine cerebral ventricular zone. J. Neurocytol *21*, 185–197.

Tole, S., Kaprielian, Z., Ou, S.K., and Patterson, P.H. (1995). FORSE-1: a positionally regulated epitope in the developing rat central nervous system. J. Neurosci. *15*, 957–969.

Yu, T.S., Dandekar, M., Monteggia, L.M., Parada, L.F., and Kernie, S.G. (2005). Temporally regulated expression of Cre recombinase in neural stem cells. Genesis *41*, 147–153.

Ziegler, A.N., Chidambaram, S., Forbes, B.E., Wood, T.L., and Levison, S.W. (2014). Insulin-like growth factor-II (IGF-II) and IGF-II analogs with enhanced insulin receptor-a binding affinity promote neural stem cell expansion. J. Biol. Chem. *289*, 4626–4633.

Ziegler, A.N., Feng, Q., Chidambaram, S., Testai, J.M., Kumari, E., Rothbard, D.E., Constancia, M., Sandovici, I., Cominski, T., Pang, K., et al. (2019). Insulin-like growth factor II: an essential adult stem cell niche constituent in brain and intestine. Stem Cell Rep. *12*, 816–830.